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Squid hnRNP Protein Promotes Apical Cytoplasmic Transport and Localization of *Drosophila* Pair-Rule Transcripts

Sabbi Lall,*§ Helen Francis-Lang,*‖ Antonin Flament,* Amanda Norvell,† Trudi Schüpbach,† and David Ish-Horowicz*‡

*Developmental Genetics Laboratory
Imperial Cancer Research Fund
Lincoln's Inn Fields
London WC2A 3PX
United Kingdom

†Howard Hughes Medical Institute
Department of Molecular Biology
Princeton University
Princeton, New Jersey 08544

Summary

***Drosophila melanogaster* pair-rule segmentation gene transcripts localize apically of nuclei in blastoderm embryos. This might occur by asymmetric (vectorial) export from one side of the nucleus or by transport within the cytoplasm. We have followed fluorescently labeled pair-rule transcripts postinjection into *Drosophila* embryos. Naked, microinjected *fushi tarazu* (*ftz*) transcripts do not localize in blastoderm embryos, indicating that cytoplasmic mechanisms alone are insufficient for apical targeting. However, prior exposure of *ftz* to *Drosophila* or human embryonic nuclear extract leads to rapid, specific, microtubule-dependent transport, arguing against vectorial export. We present evidence that *ftz* transcript localization involves the Squid (Hrp40) hnRNP protein and that the activity of hnRNP proteins in promoting transcript localization is evolutionarily conserved. We propose that cytoplasmic localization machineries recognize transcripts in the context of nuclear partner proteins.**

Introduction

It is increasingly appreciated that many mRNA transcripts localize to specific subregions of the cytoplasm (reviewed in Davis, 1997; Steward, 1997; Bashirullah et al., 1998). A common function for localization may be to control the site of translation, thereby helping target proteins to appropriate intracellular environment, as has been demonstrated for maternal *Drosophila* transcripts whose localization during oogenesis establishes axial coordinates for embryonic development (references below and Berleth et al., 1988; Ephrussi et al., 1991; Kim-Ha et al., 1991) and for targeting of β-*actin* transcripts to the leading edge of migrating fibroblasts (Kislauskis et al., 1994). Transcripts also localize in a variety of other animals and cell types: *Vg1* (in *Xenopus* oocytes), *myelin basic protein* (*MBP*; in oligodendrocytes), and *Ash1* (yeast) (Trapp et al., 1987; Kislauskis et al., 1993; Long et al., 1997; Takizawa et al., 1997).

Most cytoplasmic transcripts localize by selective cytoplasmic transport. Direct evidence for this mechanism comes from the localization of various mRNAs following injection into the cell cytoplasm. *Vg1* transcripts injected into stage III *Xenopus* oocytes localize tightly to the vegetal cortex (Yisraeli and Melton, 1988). Injected *oskar* (*osk*) transcripts accumulate correctly at the posterior pole of *Drosophila* oocytes, although localization may be due to cytoplasmic streaming rather than to active transport (Glotzer et al., 1997), and *MBP* mRNA localizes following injection into cultured oligodendrocytes (Ainger et al., 1993).

In these cases, localization depends on microtubules and/or actin microfilaments. For example, *Vg1*, *actin*, *bicoid* (*bcd*), and *MBP* transcripts are associated with the detergent-insoluble cytoskeleton (Pondel and King, 1988; Yisraeli et al., 1990; Ainger et al., 1993; Pokrywka and Stephenson, 1994). Functional experiments involving microtubule-depolymerizing drugs show that *Drosophila* oocyte transcripts and *MBP* and *Vg1* mRNAs all depend on an intact microtubule network in order to localize (Yisraeli et al., 1990; Pokrywka and Stephenson, 1991; Pokrywka and Stephenson, 1995; Carson et al., 1997). Actin microfilaments are also required: microfilament-disrupting drugs and mutational analysis reveal that *actin* mRNA and *Ash1* transcript require actin structures and actin-associated proteins in order to localize correctly (Sundell and Singer, 1991; Takizawa et al., 1997). *osk* mRNA localization is disrupted by cytoplasmic *Tropomyosin II* mutations, another actin-binding protein (Erdélyi et al., 1995; Tetzlaff et al., 1996).

One class of localized transcripts is encoded by the zygotic *Drosophila* pair-rule genes (including *fushi tarazu* [*ftz*] and *hairy* [*h*]), which are required to establish reiterated (segmental) embryonic pattern. Pair-rule transcripts are transcribed in the syncytial blastoderm embryo as seven distinct transverse stripes along the anteroposterior axis. At this stage, a monolayer of nuclei at the embryonic surface subdivides the cortical cytoplasm ("periplasm") into apical and basal compartments (Figure 1A). Most zygotic transcripts do not localize to a specific periplasmic compartment in blastoderm embryos, but pair-rule transcripts accumulate exclusively in the apical periplasm (Figure 1A; Hafen et al., 1984; Ingham et al., 1985; Kilcherr et al., 1986; Davis and Ish-Horowicz, 1991). The function of this localization is not yet established, although it may serve to restrict protein diffusion within the syncytial embryo (Davis and Ish-Horowicz, 1991).

Several lines of evidence have argued against pair-rule transcripts localizing by cytoplasmic transport. First, pair-rule mRNAs are not detectable in the basal cytoplasm, even after extensive overstaining or stabilization of transcripts (Hafen et al., 1984; Edgar et al., 1986; Davis and Ish-Horowicz, 1991; Davis et al., 1993). Second, pair-rule transcripts are extremely unstable

‡To whom correspondence should be addressed (e-mail: d.horowicz@icrf.icnet.uk).
§Present address: Department of Organismal Biology and Anatomy, Howard Hughes Medical Institute, University of Chicago, Chicago, Illinois 60637.
‖Present address: Exelixis Pharmaceuticals Inc., 260 Littlefield Ave., South San Francisco, California 94080.

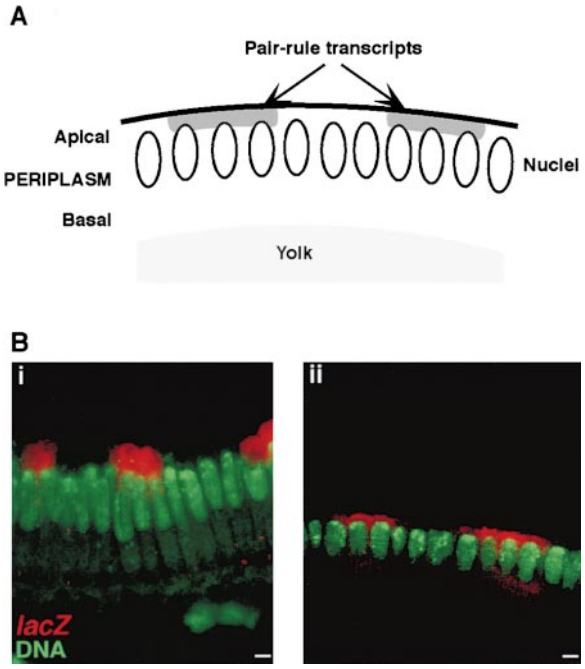

Figure 1. One Hundred Fifty Bases of the *ftz* 3′UTR Are Sufficient for Apical Targeting

(A) Diagram of transcript localization in the cortical cytoplasm of the *Drosophila* blastoderm embryo showing nuclear monolayer subdividing cortical cytoplasm into apical and basal compartment, and restriction of pair-rule transcripts to apical periplasm.

(B) Apical localization of a heterologous transcript containing only 150 bases of the *ftz* 3′UTR sequence. In situ hybridization demonstrates apical localization of hybrid *lacZ-ftz*3′UTR transcripts. (i) *lacZ* transcripts (red) generated by the *FG2* transgene carry the entire *ftz* 3′UTR and accumulate apical of blastoderm nuclei (OliGreen; Molecular Probes, OR). (ii) *LTf2* transcripts carry only 150 bases of the *ftz* 3′UTR yet also localize apically. Thus, a small portion of the large *ftz* 3′UTR is sufficient for localization.

($t_{1/2} \approx 6.5$ min; Edgar et al., 1986), so they are more likely to localize directly and rapidly. Stabilizing the transcripts does not prevent localization, showing that their selective accumulation is not due to selective degradation of basal transcripts (Edgar et al., 1986).

These observations have led to the proposal that pair-rule transcripts of *Drosophila* localize directly (i.e., by selective [vectorial] export through one side of the nuclear envelope) (Davis and Ish-Horowicz, 1991; Davis et al., 1993; Francis-Lang et al., 1996). Further evidence for this model comes from experiments examining aneuploid blastoderm embryos, in which pair-rule transcripts localize apically of displaced internalized nuclei (Francis-Lang et al., 1996), indicative of linkage between the nucleus and sites of transcript localization. Evidence connecting transcription and localization comes from studies showing that *bcd* transcripts localize apically only in cells where they are being synthesized. *bcd* transcripts are made in nurse cells, before being transported into the adjacent oocyte where they localize anteriorly. Maternal *bcd* transcripts localize apically of nuclei in nurse cells but not in the mature egg (St Johnston et al., 1989). Zygotic transcripts with a *bcd* 3′-untranslated region (3′UTR) localize apically in blastoderm embryos (Davis and Ish-Horowicz, 1991). Together, these results indicate that the *bcd* apical localization signal only operates when coupled to synthesis (Davis et al., 1993).

Despite the above arguments, it has not been possible to test the vectorial export model directly because normal transcripts do not accumulate to detectable levels en route from their sites of transcription to the nuclear envelope (e.g., Zachar et al., 1993). In particular, pair-rule transcripts could localize by very rapid cytoplasmic transport along uncharacterized routes linked to the nuclear periphery.

In this paper, we define a localization mechanism for pair-rule transcripts using a new mRNP injection assay. We show that synthetic, fluorescently tagged *ftz* transcripts are unable to localize after injection into blastoderm embryos but that preincubation with nuclear protein extracts leads to rapid and specific apical localization dependent on an intact microtubule cytoskeleton. We show that this activity can be attributed to the *Drosophila* Squid hnRNP protein (Sqd; Hrp40; Kelley, 1993; Matunis et al., 1994), which selectively binds to the *ftz* 3′UTR. Related human hnRNP proteins can substitute for Sqd activity, indicating that the localization-promoting activity of hnRNP proteins has been evolutionarily conserved. Our results argue that pair-rule transcripts localize by cytoplasmic transport as RNP complexes with coexported nuclear proteins.

Results

Injected *ftz* Transcripts Are Not Specifically Targeted in Blastoderm Embryos

Previous studies have shown that the 3′UTRs of pair-rule transcripts are necessary and sufficient to target transcripts apically (Davis and Ish-Horowicz, 1991). In the case of *ftz*, the localization signal resides within a 1.3 kb region of the *ftz* gene, which includes the *ftz* 3′UTR (Davis and Ish-Horowicz, 1991). We defined this signal more precisely using germline transformation and found that hybrid *lacZ-ftz*3′UTR transcripts with 205 bp of *ftz* 3′ genomic sequence are apically localized (*LTf2*; Figure 1B; Experimental Procedures). Transcripts that lack the last 53 bases of the 3′UTR fail to localize apically (data not shown), showing that the *ftz* 3′UTR is necessary and sufficient to target a heterologous transcript apically.

Maternal *Drosophila* and *Xenopus* transcripts that localize via cytoplasmic mechanisms also have localization signals in the 3′UTR (for example, Macdonald and Struhl, 1988; Mowry and Melton, 1992; Kim-Ha et al., 1993; Gavis et al., 1996; Bashirullah et al., 1998). Thus, endogenous pair-rule mRNAs might localize similarly, despite previous indirect evidence for a nuclear mechanism (see Introduction). We examined this possibility directly by testing whether transcripts injected into the blastoderm cytoplasm can localize specifically; if nuclear events are essential for apical accumulation of pair-rule transcripts, *ftz* mRNAs that have not been exposed to such an environment should not localize.

To check localization, we directly visualized transcripts that had been labeled with fluorescent tags (Experimental Procedures and Glotzer et al., 1997). Capped, polyadenylated transcripts incorporating aminoallyl-UTP were synthesized in vitro and chemically labeled with

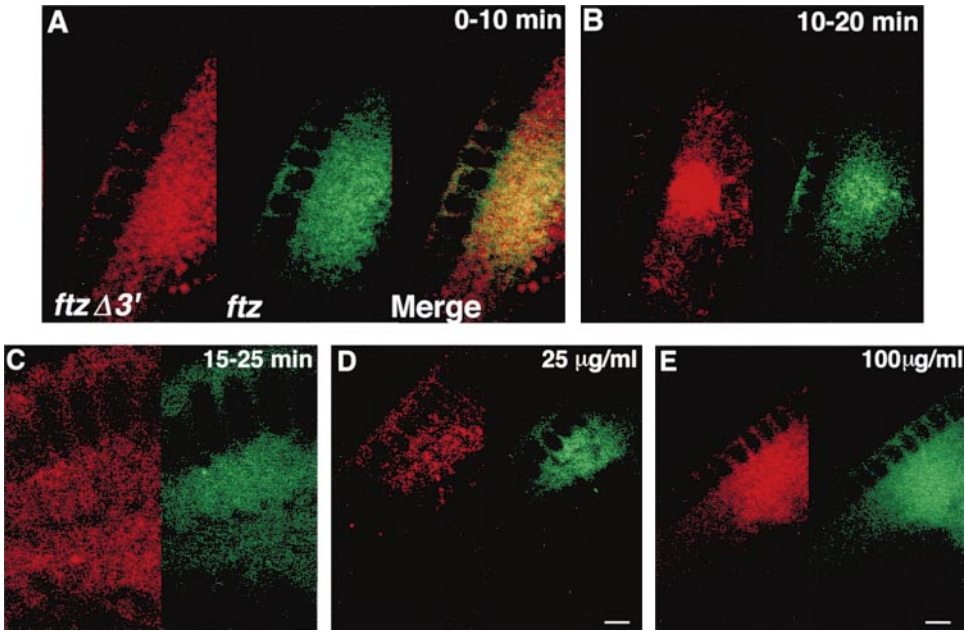

Figure 2. Injected *ftz* and *ftzΔ3′* Transcripts Colocalize in Blastoderm Embryos

In vitro synthesized *ftz* (green) and *ftzΔ3′* (red) transcripts were coinjected (100 μg/ml; A–C and E) into the basal periplasm of blastoderm embryos and incubated for 0–10 min (A), 10–20 min (B), or 15–25 min (C) after injection. No qualitative differences are observed between the two types of transcript; they do not form particles or accumulate apically. (D and E) Transcripts behave similarly when injected at 25 μg/ml, indicating that the failure to localize is not due to saturation of the machinery. Embryos were incubated for 5–15 min after injection.

either fluorescein (FITC) or Rhodamine (Rh). We excluded effects of diffusion and variability between individual injected embryos by comparing movement of coinjected wild-type *ftz* transcripts with that of truncated (*ftzΔ3′*) transcripts that are incapable of apical localization. FITC-labeled *ftz* and Rh-labeled *ftzΔ3′* transcripts were injected into the basal periplasm during nuclear cleavage cycles 13 or 14, when endogenous pair-rule transcripts are restricted to the apical periplasm. Localization of the RNAs was examined by confocal microscopy 0–30 min after injection (see Experimental Procedures).

Initial experiments showed that both transcripts are short lived, in accord with in vivo measurements of *ftz* transcript half-life (Edgar et al., 1986). Thus, the transcripts do not persist long enough to test for selective localization. To overcome this problem, we coinjected cycloheximide (equivalent intracellular concentration of ~20 μg/ml), which has previously been shown to stabilize endogenous pair-rule transcripts without affecting their localization (Edgar et al., 1986). Under these conditions, injected *ftz* and *ftzΔ3′* transcripts are still readily detectable 30 min or more after injection (e.g., Figure 2C). We find no evidence of selective localization of the full-length *ftz* transcripts (Figure 2). In essentially all embryos (>99%; $n > 400$), full-length *ftz* transcripts fail to accumulate selectively in the apical periplasm; both injected transcripts diffuse out from the site of injection and colocalize for at least 30 min (Figures 2A–2C). Thus, injected *ftz* transcripts are unable to mimic the apical localization shown by endogenous pair-rule and reporter transcripts, indicating that purely cytoplasmic mechanisms are insufficient to account for pair-rule transcript localization.

Failure of Injected *ftz* Transcripts to Localize Is Not Due to Tagging or to Saturation of the Endogenous Localization Machinery

Injected full-length *ftz* transcripts could fail to localize because fluorescent labeling perturbs transcript behavior (e.g., by inhibiting binding of proteins to the localization signal). To exclude this possibility, we tested the ability of labeled *ftz* transcripts to induce pattern abnormalities in late syncytial blastoderm embryos (cf. Jiménez et al., 1996). Tagged and untagged *ftz* transcripts injected into late syncytial blastoderm embryos are equally effective at causing local pair-rule phenotypes (reciprocal to those of *ftz* mutant embryos), akin to those due to generalized ectopic *ftz* expression (Figures 3A and 3B; Table 1; Struhl, 1985; Ish-Horowicz et al., 1989). These results indicate that labeling does not affect translatability of transcripts. Labeling also does not affect the ability of *bcd*-3′UTR transcripts to localize or recruit proteins (Ferrandon et al., 1994); injected, labeled *bcd*-3′UTR transcripts behave like unlabeled transcripts, forming large perinuclear particles that also incorporate Staufen protein (Figure 3C).

Failure of injected transcripts to localize is also not due to saturation of the endogenous localization machinery. Reducing the concentration of injected transcripts to approximately endogenous levels does not lead to selective localization (Figures 2D and 2E). In any case, the localization machinery has considerable surplus capacity: the very high levels of pair-rule transcript that accumulate following mRNA stabilization by cycloheximide all localize apically (Edgar et al., 1986), and endogenous *h* and *ftz* transcripts localize normally in injected embryos (data not shown). Together, these

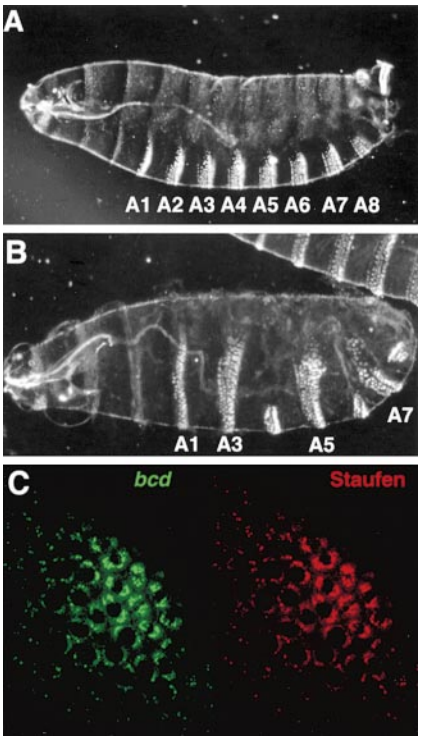

Figure 3. Fluorochrome-Labeled *ftz* Transcripts Are Functional In Vivo

(A) Wild-type cuticle. A, ventral abdominal denticle belts.
(B) Embryo injected with 200 μg/ml FITC-*ftz* mRNA, showing complete deletion of A2 and reduction of A8, A6, and A4, the reciprocal pair-rule phenotype to that of *ftz* mutant embryos.
(C) Labeled *bcd*-3′UTR RNA forms particles (green) around nuclei and recruits Staufen protein (red) from the excess pool.

results indicate that cytoplasmic factors required for anchoring or transporting pair-rule transcripts are available in excess, to be used for the localization of all apical transcripts.

ftz Transcripts Localize Apically following Exposure to Nuclear Extracts

Naked pair-rule transcripts could be unable to localize either because localization depends on vectorial nuclear export, or because transcripts need prior exposure to a nuclear environment for apical targeting (e.g., to recruit nuclear proteins that are subsequently required for localization in the cytoplasm). To distinguish between these alternatives, we tested whether nuclear proteins might promote cytoplasmic localization of pair-rule transcripts.

We exposed FITC-*ftz* and Rh-*ftz*Δ*3′* to *Drosophila* embryonic nuclear extracts and coinjected the "preincubated transcripts" with cycloheximide into the basal periplasm of cycle 14 blastoderm embryos (Experimental Procedures). Strikingly, preincubated FITC-*ftz* transcripts specifically accumulate in the apical periplasm within 10 min of injection (60/60 embryos; 10–20 min postinjection), whereas *ftz*Δ*3′* transcripts remain unlocalized (Figure 4A). Nuclear extract is much more active than control proteins in promoting apical transcript localization. A control RNA-binding protein (GST-PABP) is also largely inactive in localizing *ftz* transcripts (see Figure 6D). BSA shows some localization activity, but only at very high concentrations (Figure 4E). Together, our results indicate that nuclear extracts include specific factors required to localize pair-rule transcripts in blastoderm embryos.

Preincubated *ftz* transcripts localize in apical caps above the nuclei, thereby differing slightly from endogenous transcripts that localize as a continuous stripe domain (Davis and Ish-Horowicz, 1991). A further difference is that the accumulations of preincubated transcripts appear more particulate than that of endogenous transcripts, although this may reflect differences in expression levels or detection methods. In any case, the efficiency of localization is high, and little residual transcript remains at the site of injection. Thus, nuclear extracts include factors that specifically promote apical localization of preincubated *ftz* transcripts.

Endogenous *ftz* transcripts are never observed in the basal periplasm (Hafen et al., 1984; Davis and Ish-Horowicz, 1991), indicating that localization by a cytoplasmic mechanism should be extremely rapid. Efficient localization occurs in all embryos 4–5 min after injection (50/50, Figure 4B), and 50% of embryos display apical localized transcripts 2–2.5 min after injection (15/30; Figure 4C). Thus, preincubated transcripts also localize rapidly. We do not observe localization at earlier time points (0–1 min; 0/45), although we find particles of FITC-*ftz* transcripts that may represent intermediates of cytoplasmic targeting (11/45; Figure 4D). Localization of preincubated transcripts thereafter must be a speedy process.

Localization of Preincubated *ftz* Is Dependent upon Microtubules but Independent of Microfilaments

Injected transcripts are essentially stable under the conditions of our assay, so specific localization of the preincubated FITC-*ftz* transcripts should be the consequence

Table 1. FITC Labeling Does Not Impair the Ability of Injected *ftz* Transcripts to Regulate Segmentation

Transcript Injected	Wild-Type Cuticles (%)	Denticle Belt Phenotypes (%)		n
		Even Numbered	Odd Numbered	
Unmodified *ftz* (100 μg/ml)	80	20	0	61
FITC-*ftz* (100 μg/ml)	82	18	0	148
FITZ-*ftz* (200 μg/ml)	67	32	1	100
Buffer alone	97	0	3	27

Embryos were injected with transcripts, and larval cuticle was examined for segmentation defects in the ventral denticle belts. Phenotypes observed were specific to even-numbered belts, as occurs with global *ftz* overexpression (Ish-Horowicz et al., 1989). FITC-labeled and unlabeled *ftz* induce phenotypes at the equal frequencies.

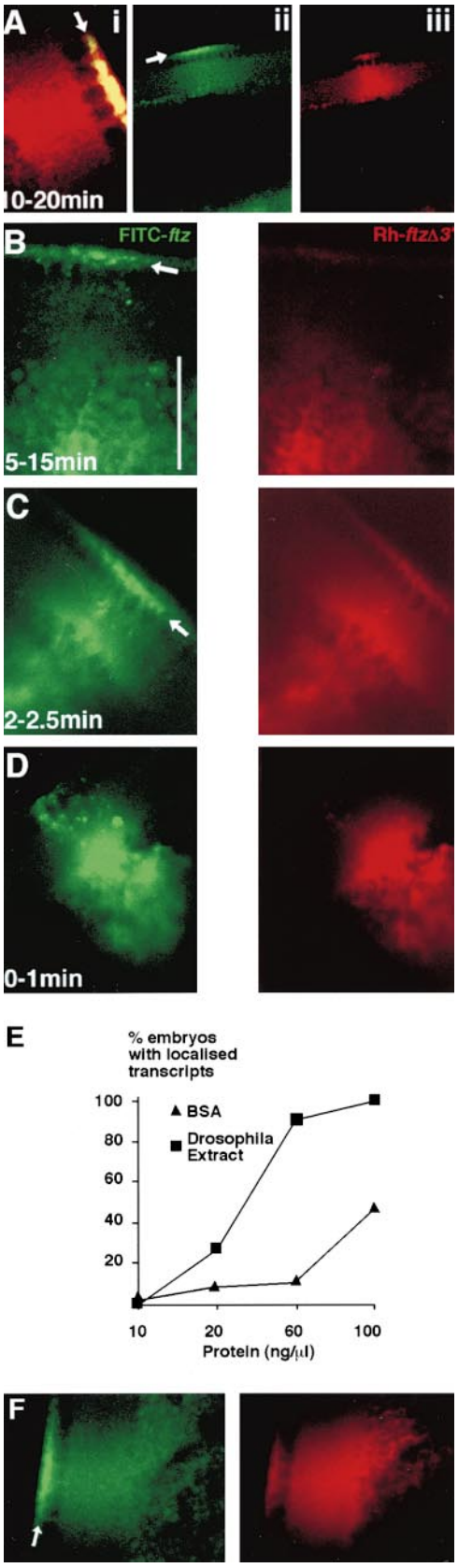

Figure 4. *Drosophila* Nuclear Extracts Promote Localization of Pair-Rule Transcripts

FITC-*ftz* (green) and Rh-*ftz*Δ*3′* (red) were preincubated with *Drosophila* embryonic nuclear extract; the FITC-*ftz* transcripts localize rapidly and specifically apically (arrows).

(A) Within 10–20 min after injection, FITC-*ftz* transcripts (green/yellow; i and ii) have localized in apical caps, whereas Rh-*ftz*Δ*3′* transcripts diffuse (i and iii).

(B) Localization of FITC-*ftz* transcript occurs within 5 min, in contrast to nonlocalized Rh-*ftz*Δ*3′* transcripts. Transcripts from the basal periplasm (but not from the yolk [vertical white bar]) have been transported apically.

(C) Within 2–2.5 min, much of the injected FITC-*ftz* transcripts has localized, unlike the Rh-*ftz*Δ*3′* transcripts.

(D) Within 1 min, preincubated FITC-*ftz* transcripts show specific particle formation and some movement away from the pool of coinjected transcript.

(E) Relative activities of nuclear extract and BSA in promoting apical transcript localization. Standard deviations are less than 10%.

(F) Mammalian nuclear extract also promotes apical localization of preincubated FITC-*ftz* transcript. FITC-*ftz* that has been exposed to 50 ng/μl TIG-3 nuclear extract localizes apically (arrow), when compared to Rh-*ftz*Δ*3′*.

of a selective transport system. To define the transport mechanism further, we tested its dependence on an intact cytoskeleton. Early cycle 14 embryos were injected with 2 μg/ml of colcemid and, 10 min later, injected with preincubated FITC-*ftz* transcript mixture. Localization is almost completely disrupted (21/22 embryos) such that the behavior of FITC-*ftz* resembles that of coinjected Rh-*ftz*Δ*3′* (Figure 5A). This inhibition of localization indicates that pair-rule transcript localization depends on an intact microtubule cytoskeleton.

By contrast, preincubated FITC-*ftz* transcripts still localize apically in embryos that have been coinjected with 10 μg/ml of Cytochalasin B ($n = 30$; Figures 5B and 5C). This concentration has previously been shown to disrupt actin-dependent processes such as anchoring of nuclei to the cortex (Zalokar and Erk, 1976; Foe and Alberts, 1983) and, indeed, causes displacement of nuclei into the basal periplasm (e.g., Figure 5C). Thus, processes disrupted by Cytochalasin B are not required for apical localization of preincubated FITC-*ftz* transcripts.

Nuclear Factors Promoting Cytoplasmic Transcript Targeting Are Evolutionarily Conserved

It is possible that factors involved in transcript targeting are evolutionarily conserved. Rat neuronal *tau* transcripts localize to the vegetal pole when injected into *Xenopus* oocytes (Litman et al., 1996). Also the mammalian ZBP-1 protein, which binds to the localization signal of β-*actin* transcripts (Ross et al., 1997), is homologous to the VERA/VBP protein, which recognizes the *Vg1* localization signal in *Xenopus* oocytes (Deshler et al., 1998; Havin et al., 1998). Thus, we examined whether a human nuclear extract from TIG-3 cells (human fetal lung fibroblasts) can also promote pair-rule transcript localization (Experimental Procedures). Figure 4F shows that preexposed FITC-*ftz* transcripts specifically localize apically (21/21 at 50 ng/μl protein), indicating that human nuclear extracts indeed promote pair-rule transcript localization. This degree of activity is higher than that of an equivalent *Drosophila* extract (35% embryos; Figure 4E), although the proportion of localized transcripts within each embryo appears lower with human extracts.

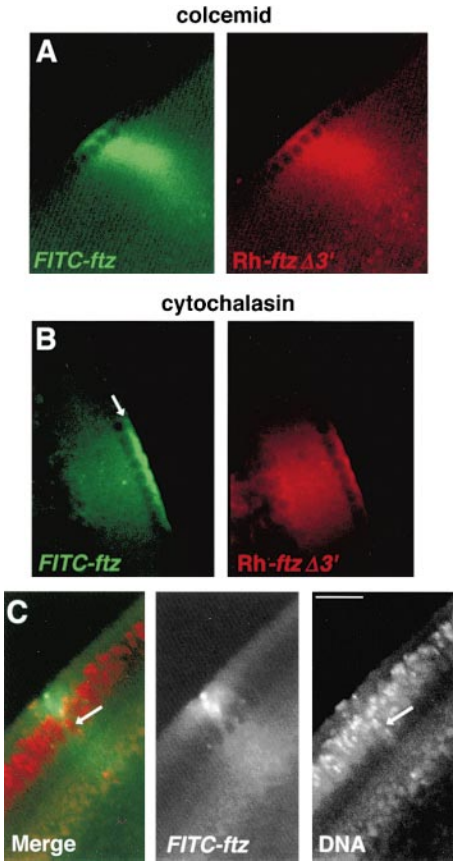

Figure 5. Microtubules, but Not the Actin Cytoskeleton, Are Required for the Apical Localization of Preincubated *ftz* Transcripts

(A) Embryos treated with colcemid are unable to localize FITC-*ftz* transcripts (green), which now behave like control Rh-*ftzΔ3′* transcripts (red).

(B) In embryos coinjected with Cytochalasin B, FITC-*ftz* transcripts still localize specifically apically (green; arrow), unlike Rh-*ftzΔ3′* transcripts (red).

(C) Transcripts (green) remain apical of an internalizing nucleus (arrow), stained for DNA with daunomycin (red).

In any case, an activity that promotes pair-rule transcript localization is conserved between flies and humans.

Squid and Related hnRNP's Facilitate Localization of Pair-Rule Transcripts

For several reasons, we considered the possibility that the nuclear factors facilitating localization are hnRNP proteins. First, hnRNP's are well conserved between flies and humans. Second, the nuclear factors that facilitate localization appear to function in the cytoplasm and therefore must shuttle between nucleus and cytoplasm, as do hnRNP's (Piñol-Roma and Dreyfuss, 1992; Visa et al., 1996; reviewed in Piñol-Roma, 1997; Mattaj and Englmeier, 1998). Third, the *Drosophila* Sqd hnRNP protein, a homolog of the mammalian hnRNP-A/B proteins, is required for *gurken* (*grk*) transcripts to localize during oogenesis (Kelley, 1993; Neuman-Silberberg and Schüpbach, 1993; Matunis et al., 1994). Finally, hnRNP-A2 has been shown to bind to a 3′UTR sequence required for the localization of *MBP* transcripts in rat oligodendrocytes (Hoek et al., 1998).

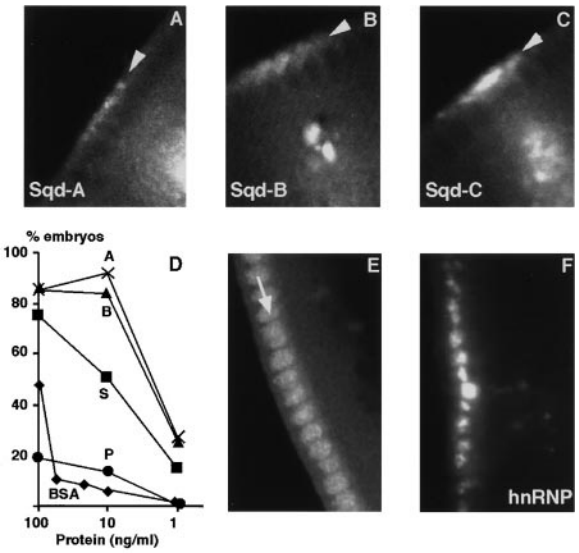

Figure 6. Squid and Human hnRNP Protein Homologs Promote Apical Localization of Injected *ftz* Transcripts

(A–C) FITC-*ftz* transcripts localize apically (arrowhead) following preincubation with 0.01 μg/μl of GST fused to Sqd-A, -B or -S. $n > 30$ for each isoform.

(D) Titration of localization activities. The three isoforms of Sqd (A, B, and S) show significantly increased activity in the localization assay compared to BSA and human PABP (P) controls. Standard deviations are less than 10%, except GST-SqdB at 1 ng/ml, which was more variable (± 25%).

(E) Immunofluorescence shows that Sqd is present throughout blastoderm nuclei (arrow), consistent with its association with *ftz* and other transcripts before and during export. Sqd is not significantly enriched apically.

(F) Apical *ftz* transcript localization driven by preincubation with human hnRNP-B. The other two hnRNP proteins tested behave similarly: transcripts localized in 18/24 embryos, -A1; 17/20, -A2; 27/31, -B.

To test whether Sqd protein promotes cytoplasmic transport, we preincubated transcripts with each of the three Sqd protein isoforms (fusions to glutathione-S-transferase [GST], expressed in bacteria; Experimental Procedures) and injected them into blastoderm embryos. All three fusion proteins are active in promoting apical localization. Thus, preexposure to any of the Sqd isoforms leads to localization of labeled *ftz* but not *ftzΔ3′* transcripts (Figures 6A–6C). Control proteins, including BSA and a GST fusion to the polyA-binding protein (PABP), are about 100-fold less active at limiting concentrations (Figure 6D). The slightly lower activity of SqdB in our assay is probably not significant, although it is notable that expression of this isoform is incapable of supporting *grk* transcript localization during oogenesis (Norvell et al., 1999). Association of *ftz* transcripts with Sqd is very rapid, being essentially complete within the 2 min period required to establish injections (data not shown).

We also tested whether the ability of hnRNP proteins to promote transcript localization has been evolutionarily conserved. *ftz* transcripts preincubated with each of the human hnRNP-A1, -A2 and -B proteins (gifts of Dr. A. Krainer) localize apically (Figure 6F), showing that the activity of this class of A/B hnRNP proteins is conserved between *Drosophila* and humans.

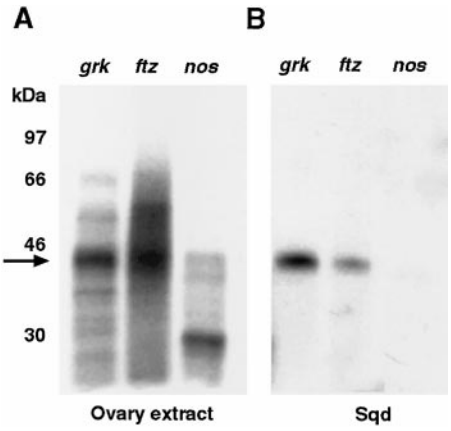

Figure 7. UV Cross-Linking Demonstrates that Sqd Protein Interacts Selectively with the *ftz* 3′UTR

(A) SDS-PAGE of whole-cell ovarian extracts following labeling with ^{32}P-labeled *grk* or *ftz* 3′UTR probes, which bind Sqd (42 kDa; arrow), or a control *nanos*+*3*3′UTR probe, which does not (lane 3; Gavis et al., 1996).

(B) Gel of cross-linked, labeled Sqd purified by immunoprecipitation, showing that the 42 kDa activity that binds the *ftz* 3′UTR is indeed Sqd.

Sqd Is a Major *ftz* 3′UTR Binding Activity in Ovary Extracts

Immunostaining of early embryos shows that Sqd is indeed present in blastoderm nuclei (Figure 6E), as expected if Sqd is the major in vivo localizing activity. Unfortunately, we cannot test directly whether Sqd is required to localize pair-rule transcripts because strong *sqd* mutant eggs are unfertilized. However, we determined whether Sqd selectively recognizes the *ftz* 3′UTR by examining protein extracts from ovaries, the major source of Sqd in blastoderm embryos.

Proteins that bind to *ftz*-3′UTR transcripts were labeled by UV cross-linking to ^{32}P-labeled transcripts and visualized following gel electrophoresis (Norvell et al., 1999; Experimental Procedures). Figure 7A shows that *ftz*-3′UTR transcripts label a predominant 42 kDa protein in *Drosophila* ovary extracts, the same size as Sqd and as the activity labeled by the *grk*-3′UTR (Figure 7A). The 42 kDa protein is indeed Sqd, being immunoprecipitated by anti-Sqd antibodies and only weakly labeled by a control *nanos*-3′UTR transcript (Figure 7B). Thus, Sqd binds specifically to the *ftz*-3′UTR and represents a major such activity in oocyte extracts. Other proteins are also labeled by *ftz*, including one at ~55–60 kDa; because the experiments are performed with whole-cell extracts, these are not necessarily nuclear. Additional *ftz*-binding proteins could be involved in transcript localization, or they may mediate other aspects of *ftz* transcripts (e.g., their instability).

Discussion

Our experiments show that naked pair-rule transcripts fail to localize following injection into the blastoderm cytoplasm, implying that prior exposure to a nuclear environment is necessary for transcript localization. We confirmed this view by using a novel mRNP injection assay to reveal that prior exposure to nuclear proteins promotes apical transcript localization, thereby identifying a requirement for nuclear transcript-binding proteins for subsequent cytoplasmic transport and showing that pair-rule transcripts can localize exclusively by cytoplasmic transport.

Previous proposals that pair-rule transcripts localize by vectorial nuclear export rested largely on the absence of evidence for their cytoplasmic transport. However, our demonstration that pair-rule transcripts can undergo cytoplasmic transport renders the vectorial export model unlikely. The ability of human hnRNP proteins to promote localization and the uniform expression of Sqd within blastoderm nuclei (Figure 6E) also argue against a vectorial export mechanism (although we cannot exclude its operating as a second, redundant mechanism).

Localization of preincubated pair-rule transcripts requires an intact microtubule cytoskeleton and is likely to be mediated by microtubule-dependent motors. In wild-type blastoderm embryos, each nucleus is indeed capped by an apical bundle of microtubules that could serve as a framework for transcript transport. Apical transport along these microtubules would require transport by a dynein-like motor (Sullivan and Theurkauf, 1995). However, pair-rule transcripts can localize elsewhere in aneuploid blastoderm embryos. In *3L*$^{-}$ embryos, some nuclei become internalized and lack associated apical microtubules (Francis-Lang et al., 1996); nevertheless, transcripts accumulate adjacent to and apical of these nuclei, raising the possibility that pair-rule transcripts run along a different, minority class of microtubules.

After transport, pair-rule transcripts are anchored to the cytoskeleton, as shown by their lack of diffusion in blastoderm embryos (Edgar et al., 1987). However, the transcripts still localize apically of nuclei displaced from the periphery of the embryo following Cytochalasin B treatment (Figures 5B and 5C), indicating that transcript attachment sites differ from those of nuclei and are perhaps not microfilament based. In the latter case, they would differ from those already implicated in transcript localization (e.g., Yisraeli et al., 1990; Taneja et al., 1992).

Pair-rule transcripts are very unstable in vivo, so their transport should be very rapid. This is indeed the case for injected *ftz* transcripts, explaining why endogenous localization intermediates have been difficult to observe in vivo. Within 1 min, injected transcripts are recruited into granules resembling those observed in other microtubule-dependent mRNA injection assays (e.g., Ainger et al., 1993; Ferrandon et al., 1994, 1997; Carson et al., 1997; Glotzer et al., 1997), and the bulk of injected transcripts are apically localized 1–2 min later (i.e., much more quickly than injected *osk* and *Vg1* transcripts, which localize over periods of hours or days, respectively) (Yisraeli and Melton, 1988; Glotzer et al., 1997). Injected transcripts can localize 10–25 μm away from the injection site, equivalent to a transport rate of 10 μm/min, and approximately equal to the speed of localizing *MBP* transcripts and of induced transcripts in electrical stimulated rat neurons (~5–12 μm/min; Ainger et al., 1993; Wallace et al., 1998).

Our results provide very strong evidence that the Sqd hnRNP protein is a major factor acting in vivo in localizing *ftz* transcripts. Sqd is present in blastoderm nuclei,

where it can associate with nascent pair-rule transcripts before export (Figure 6E). Our result that Sqd is the predominant in vitro *ftz* 3′UTR binding activity (Figure 7A) shows that Sqd recognizes the *ftz* 3′UTR directly. We estimate that the Sqd in nuclear extracts can account for ~20%–35% of the activity in the extracts (see Experimental Procedures), more if bacterially expressed GST-Sqd is less active than endogenous Sqd. Sqd binding to *ftz* transcripts is very efficient; we estimate that a 4-fold molar excess of Sqd in the incubations is sufficient to drive significant levels of transcript localization (Experimental Procedures), consistent with one Sqd molecule promoting localization of a single transcript molecule. Together, these results argue strongly that Sqd acts in vivo. Nevertheless, other hnRNP proteins may also have localization activity.

Injected naked transcripts remain predominantly basal (e.g., Figure 2C), arguing that Sqd is required for active transport, although it remains possible that Sqd functions in both transport and anchorage. The efficiency of Sqd binding makes it unlikely that Sqd acts solely in the nucleus to remodel the *ftz* 3′UTR conformation and that it is not normally present on the cytoplasmic transcript. We favor its comprising part of a localization signal such that the transport machinery recognizes a Sqd-containing RNP complex. Future experiments using our rapid mRNP injection assay will allow us to define more precisely the structural basis of the 3′UTR apical localization signals of *ftz* and other localizing transcripts and, thereby, to determine the functional significance of pair-rule transcript localization during segmentation. Detailed characterization of the localization signal will also permit identification of factors that selectively target Sqd/*ftz* mRNPs for apical transport.

In addition to its probable role in localizing *ftz* transcripts, Sqd is also required for localization of *grk* transcripts (Kelley, 1993; Neuman-Silberberg and Schüpbach, 1993). In *sqd* mutant embryos, *grk* transcripts are still exported from the nucleus, and the first phase of *grk* localization that establishes anteroposterior polarity appears normal; however, subsequent anterodorsal *grk* transcript localization fails. Detailed analysis of *sqd* and *fs(1)K10* mutations argues that *grk* transcript localization depends specifically on Sqd protein being present in the oocyte nucleus, implying that *grk* transcripts must associate with Sqd as nascent transcripts (Norvell et al., 1999). A requirement for nuclear Sqd in cytoplasmic *grk* transcript localization would parallel that required to localize *ftz* transcripts.

Individual human hnRNP's retain the ability to promote localization of *ftz* transcripts. Thus, the factors and machinery required for intracellular targeting of transcripts are likely to be general and highly conserved. This is also indicated by the ability of transcripts to localize in heterologous species (Litman et al., 1996) and the binding of hnRNP's to other localizing transcripts: *MBP* transcripts in rat oligodendrocytes (Hoek et al., 1998), and *Vg1* transcripts in *Xenopus* oocytes (K. Mowry, personal communication). We therefore propose that our results are not particular to localization of *ftz* transcripts and that transcripts are generally transported as RNP complexes containing nuclear proteins. This model also explains why maternal *bcd* transcripts only localize apically in transcribing cells (see Introduction). We suggest that *bcd* transcripts bind hnRNP's during synthesis in nurse cells and that this association does not persist during transport to the oocyte cytoplasm and egg maturation.

Experimental Procedures

A full account of experimental procedures can be found as supplementary material on the website <http://www.cell.com/cgi/content/full/98/2/171/DC1>.

Transcription and Labeling of Fluorescently Tagged mRNA

RNAs were transcribed from cDNAs inserted into *pBsptpA:pKS*, a modified Bluescript plasmid (Stratagene) with an A_{23} sequence inserted between the HindIII and XhoI sites. XhoI-linearized DNA was transcribed for 2 hr at 37°C with T7 polymerase in a 100 μl reaction mix (Stratagene) including 0.25 mM aminoallyl-UTP (Sigma), 2.25 mM UTP, and 1 mM ^{7}mG(5′)ppp(5′). After 15 min incubation with 40 U DNAase, RNA was extracted with phenol/$CHCl_3$, EtOH precipitated, and unincorporated nucleotides were removed on a Sephadex G50 spin-column (Boehringer-Mannheim). After reprecipitation with NH_4OAc/EtOH, 25 μg RNA was labeled using 5 mM of either [6-(fluorescein-5-(and-6)-carboxamido)hexanoic acid, succinimydyl ester] or [5-(and-6)-carboxy-X-rhodamine, succinimydyl ester] (Molecular Probes), as described by Glotzer et al., (1997) except that later experiments used 0.15 M bicarbonate buffer (pH 9). Unincorporated fluorochrome was removed using a Sephadex G50 spin-column and NH_4OAc/EtOH precipitation, and the RNA was resuspended in water. Incorporation and yield of labeled RNA were assessed by UV spectrophotometry; transcripts contained on average 1 fluorochrome molecule per 75–120 bases, typically, 15–25 fluorochromes per molecule of RNA.

Transcript Preincubation

Labeled transcript (2 μg) was exposed to varying amounts of *Drosophila* nuclear extract in 10 μl of 50 mM KCl, 2% glycerol, 20 mM HEPES, 0.5 mM EDTA, for 2–30 min on ice. Before injection, the reaction was diluted 2-fold with buffer to increase EDTA and glycerol concentrations to those of injection buffer. *ftz* transcript was also coinjected with other proteins including 50 ng/μl human extract; varying concentrations of BSA; GST-PABP, or GST-fusions of the three Sqd isoforms (Kelley, 1993) expressed in *E. coli* and purified on a glutathione-Sepharose column (Smith and Johnston, 1988); and 10 ng/μl of bacterially expressed human hnRNP-A1, -A2, or B proteins (gift of A. Krainer). Embryos were scored for transcript localization without prior identification of the injected protein.

Embryo Injections

Dechorionated 2–2.5 hr embryos (1–1.5 hr for injecting *bcd*-3′UTR) were injected through the ventral side into the dorsal basal periplasm with RNA resuspended in injection buffer (Anderson and Nüsslein-Volhard, 1984) plus cycloheximide. Unless otherwise indicated, embryos were incubated in the dark for 10–20 min before fixation. Embryos treated with Cytochalasin B and colcemid (10 μg/ml and 2 μg/ml, respectively) were injected at the onset of cycle 14 (Edgar et al., 1987). In the latter case, preincubated transcripts were injected separately, 5–10 min later, because microtubules are relatively stable in cellularizing blastoderm embryos.

To visualize injected transcripts, halocarbon oil was removed using heptane, and embryos were fixed with heptane saturated with 37% formaldehyde/phosphate-buffered saline for 20 min in the dark. Embryos were hand peeled and analyzed directly or, in the case of the *bcd*-3′UTR injection, after immunovisualization of Staufen. In all cases, embryos were mounted in "Citifluor" medium, and confocal analysis was carried out on a Leica TCS NT or a BioRad MRC600 workstation.

UV Cross-Linking Analysis and Sqd Visualization

Sqd in blastoderm embryos was detected by immunofluorescence and a mouse monoclonal antibody (8G6; Matunis et al., 1994). Sqd

in nuclear extracts was detected by Western blots on a nylon membrane (ICN) and ECL (Amersham). The combined intensities of the three Sqd bands were compared with dilutions of GST-SqdA run on the same gel. Nuclear extract (18 μg) gave a comparable signal to ~250 ng GST-Sqd, implying that 20 ng/μl extract contains ~0.25 ng/μl Sqd and shows an activity in our assay equivalent to a 1 ng/μl solution of GST-Sqd (Figures 4E and 6D). ^{32}P-UTP-labeled transcripts were made and cross-linked to oocyte extracts as previously described (Hedley and Maniatis, 1991; Norvell et al., 1999). Labeled Sqd was purified by incubation with monoclonal anti-Sqd and resolved by SDS–PAGE.

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